The Nuclear Receptors Constitutive Active/Androstane Receptor and Pregnane X Receptor Activate the Cyp2c55 Gene in Mouse Liver

Yoshihiro Konno, Hiroki Kamino, Rick Moore, Fred Lih, Kenneth B. Tomer, Darryl C. Zeldin, Joyce A. Goldstein, and Masahiko Negishi

Pharmacogenetics Section, Laboratory of Reproductive and Developmental Toxicology (Y.K., H.K., R.M., M.N.), and Laboratories of Pharmacology and Chemistry (J.A.G.), Respiratory Biology (D.C.Z.), and Structural Biology (F.L., K.B.T.), National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina

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ABSTRACT:

Mouse CYP2C55 has been characterized as an enzyme that catalyzes synthesis of 19-hydroxyeicosatetraenoic acid (19-HETE), an arachidonic acid metabolite known to have important physiological functions such as regulation of renal vascular tone and ion transport. We have now found that CYP2C55 is induced by phenobarbital (PB) and pregnenolone 16α-carbonitrile (PCN) in both mouse kidney and liver. The nuclear xenobiotic receptors constitutive active/androstane receptor (CAR) and pregnane X receptor (PXR) regulate these drug inductions: CYP2C55 mRNA was increased 25-fold in PB-treated Car(+/+ ) but not in Car(−/−) mice and was induced in Pxr(+/+ ) but not Pxr(−/−) mice after PCN treatment. Cell-based promoter analysis and gel shift assays identified the DNA sequence 1679TGAACCCAGTTGAACT−1664 as a DR4 motif that regulates CAR- and PXR-mediated transcription of the Cyp2c55 gene. Chronic PB treatment increased hepatic microsomal CYP2C55 protein and serum 19-HETE levels. These findings indicate that CAR and PXR may play a role in regulation of drug-induced synthesis of 19-HETE in the mouse.

The human CYP2C subfamily of cytochrome P450 (P450) mono-oxygenases is responsible for metabolism of many therapeutically prescribed drugs, such as phenytoin, warfarin, tolbutamide, and numerous nonsteroidal anti-inflammatory drugs, as well as the metabolism of endogenous compounds such as arachidonic acid (AA) (Goldstein and de Morais, 1994; Miners and Birkett, 1998). Fifteen murine CYP2C genes have been identified (Luo et al., 1998; DeLozier et al., 2004; Nelson et al., 2004; Wang et al., 2004), including Cyp2c29, Cyp2c36, Cyp2c37, Cyp2c38, Cyp2c39, Cyp2c40, Cyp2c44, and Cyp2c55. The expression of the Cyp2c2 genes is regulated differentially (DeLozier et al., 2004; Jackson et al., 2004, 2006; Goetz et al., 2006). For example, the Cyp2c29 and Cyp2c37 genes were up-regulated by CAR activators but not the PXR activator pregnenolone 16α-carbonitrile (PCN) (Jackson et al., 2004, 2006). CYP2C55 was characterized as an enzyme that catalyzes the biosynthesis of 19-hydroxyeicosatetraenoic acid (19-HETE) (Wang et al., 2004). A recent study showed that hepatic mRNAs for several murine CYP2C enzymes that are able to metabolize midazolam in recombinant studies were increased in Cyp3a knockout mice, particularly CYP2C55 (35-fold increase) (van Waterschoot et al., 2008). Although midazolam is thought to be metabolized primarily by the
CYP3A enzyme in Cyp3a(+/+) mice, the CYP2C enzymes were found to be the major enzymes responsible for midazolam metabolism in Cyp3a(+/−) mice as a result of their increased expression. CYP2C55 is also induced by triazole fungicides, similar to Cyp3a11, thereby mediating triazole-induced hepatotoxicity (Goetz et al., 2006). However, the molecular mechanisms responsible for the induction of the Cyp2c55 gene by therapeutic drugs have not yet been investigated.

Murine CYP2C enzymes catalyze the metabolism of AA and produce various physiologically functional eicosanoids, including cys-epoxyicosatrienoic acids (5,6-, 8,9-, 11,12-, and 14,15-EET), midchain hydroxyicosatetraenoic acids (5-, 8-, 9-, 11-, and 15-HETE), and α-terminal alcohols of AA (16-, 17-, 18-, 19-, and 20-HETE) (Capdevila et al., 2000; Zeldin, 2001; De Lozier et al., 2004). CYP2C55 showed high selectivity for 19-HETE production (Wang et al., 2004). 19-HETE has been reported to affect vascular tone and ion transport in the kidney and brain (Escalante et al., 1998; Carroll et al., 1996; Qu et al., 2001). Thus, induction of CYP2C55 by xenobiotics may have physiological effects as a result of changes in the biosynthesis of 19-HETE.

In this study, we examined whether hepatic and renal CYP2C55 mRNA was induced by the CAR agonist phenobarbital (PB) and the PXR ligand PCN in CAR- and PXR-null mice and wild-type controls. We performed cell-based promoter analyses and gel shift assays to delineate the mechanisms that regulate Cyp2c55 gene induction. Furthermore, we examined the levels of CYP2C55 protein in liver microsomes and of serum 19-HETE using Western blot analysis and liquid chromatography/tandem mass spectrometry, respectively. We found that both CAR and PXR play an essential role in regulation of the synthesis of 19-HETE by drugs.

Materials and Methods

Materials and Reagents. Dimethyl sulfoxide (DMSO), PB sodium salt, PCN, and diethylnitrosamine (DEN) were purchased from Sigma-Aldrich (St. Louis, MO). The plasmid pGL3 basic was obtained from Promega (Madison, WI). Restriction endonucleases and DNA-modifying enzymes (St. Louis, MO). The plasmid pGL3/Cyp2c55—2.5 kilobases (kb) (−2452+38), amplified sequences from mouse genomic DNA were cloned into the XhoI and HindIII sites of pGL3 basic from Promega. Primers used for amplifications were 5′-CCCTCCTAGGACGACATATTGTTGATGGAACAGAATG-3′ and 5′-AAGAGAAATCCTGCATGGTCATGTTGACTC-3′, reverse primer 5′-GAGTCACACAGACTGGGTACCTATC-3′, forward primer 5′-GGATCACAGAGATGTCATTATGATGTCATGTTGACTC-3′ and reporter plasmid pGL3/Cyp2c55—2.5 kb as a template. In the context of pGL3/Cyp2c55—2.5 kb, putative CAR and PXR binding sites, DR4 (16 bases) and DR5 (17 bases), were independently deleted using the Quick change site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) using the following primers: 5′-CATAGTTGATCCTGGGAAGCCTGAAGAGAA-3′ and 5′-TTCTCTTCTACGTCCTCCCAAGATCATTGATGTCATGTTGACTC-3′, reverse primer 5′-GAGTCACACAGACTGGGTACCTATC-3′, forward primer 5′-GGATCACACAGACTGGGTACCTATC-3′, and reporter plasmid pGL3/Cyp2c55—2.5 kb as a template. In the context of pGL3/Cyp2c55—2.5 kb, putative CAR and PXR binding sites, DR4 (16 bases) and DR5 (17 bases), were independently deleted using the Quick change site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) using the following primers: 5′-CATAGTTGATCCTGGGAAGCCTGAAGAGAA-3′ and 5′-TTCTCTTCTACGTCCTCCCAAGATCATTGATGTCATGTTGACTC-3′, reverse primer 5′-GAGTCACACAGACTGGGTACCTATC-3′, forward primer 5′-GGATCACACAGACTGGGTACCTATC-3′, and reporter plasmid pGL3/Cyp2c55—2.5 kb as a template. In the context of pGL3/Cyp2c55—2.5 kb, putative CAR and PXR binding sites, DR4 (16 bases) and DR5 (17 bases), were independently deleted using the Quick change site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) using the following primers: 5′-CATAGTTGATCCTGGGAAGCCTGAAGAGAA-3′ and 5′-TTCTCTTCTACGTCCTCCCAAGATCATTGATGTCATGTTGACTC-3′, reverse primer 5′-GAGTCACACAGACTGGGTACCTATC-3′, forward primer 5′-GGATCACACAGACTGGGTACCTATC-3′, and reporter plasmid pGL3/Cyp2c55—2.5 kb as a template. In the context of pGL3/Cyp2c55—2.5 kb, putative CAR and PXR binding sites, DR4 (16 bases) and DR5 (17 bases), were independently deleted using the Quick change site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) using the following primers: 5′-CATAGTTGATCCTGGGAAGCCTGAAGAGAA-3′ and 5′-TTCTCTTCTACGTCCTCCCAAGATCATTGATGTCATGTTGACTC-3′, reverse primer 5′-GAGTCACACAGACTGGGTACCTATC-3′, forward primer 5′-GGATCACACAGACTGGGTACCTATC-3′, and reporter plasmid pGL3/Cyp2c55—2.5 kb as a template. In the context of pGL3/Cyp2c55—2.5 kb, putative CAR and PXR binding sites, DR4 (16 bases) and DR5 (17 bases), were independently deleted using the Quick change site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) using the following primers: 5′-CATAGTTGATCCTGGGAAGCCTGAAGAGAA-3′ and 5′-TTCTCTTCTACGTCCTCCCAAGATCATTGATGTCATGTTGACTC-3′, reverse primer 5′-GAGTCACACAGACTGGGTACCTATC-3′, forward primer 5′-GGATCACACAGACTGGGTACCTATC-3′, and reporter plasmid pGL3/Cyp2c55—2.5 kb as a template. In the context of pGL3/Cyp2c55—2.5 kb, putative CAR and PXR binding sites, DR4 (16 bases) and DR5 (17 bases), were independently deleted using the Quick change site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) using the following primers: 5′-CATAGTTGATCCTGGGAAGCCTGAAGAGAA-3′ and 5′-TTCTCTTCTACGTCCTCCCAAGATCATTGATGTCATGTTGACTC-3′, reverse primer 5′-GAGTCACACAGACTGGGTACCTATC-3′, forward primer 5′-GGATCACACAGACTGGGTACCTATC-3′, and reporter plasmid pGL3/Cyp2c55—2.5 kb as a template. In the context of pGL3/Cyp2c55—2.5 kb, putative CAR and PXR binding sites, DR4 (16 bases) and DR5 (17 bases), were independently deleted using the Quick change site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) using the following primers: 5′-CATAGTTGATCCTGGGAAGCCTGAAGAGAA-3′ and 5′-TTCTCTTCTACGTCCTCCCAAGATCATTGATGTCATGTTGACTC-3′, reverse primer 5′-GAGTCACACAGACTGGGTACCTATC-3′, forward primer 5′-GGATCACACAGACTGGGTACCTATC-3′, and reporter plasmid pGL3/Cyp2c55—2.5 kb as a template. In the context of pGL3/Cyp2c55—2.5 kb, putative CAR and PXR binding sites, DR4 (16 bases) and DR5 (17 bases), were independently deleted using the Quick change site-directed mutagenesis kit (Agilen...
CYP2C55 was prepared as described previously (Wang et al., 2004). The micromoles (6 μg/lane for CYP2B10, 20 μg/lane for CYP2C55) and recombinant CYP2C55 (0.5 pmol of P450/lane) were electrophoresed in 12% Tris glycine gels, and the resolved proteins were transferred onto polyvinylidene difluoride membranes. Membranes were immunoblotted using the rabbit anti-CYP2C55 antibodies (1:1 500 dilution), rabbit anti-CYP2B10 antibodies (1: 20 000 dilution), goat anti-rabbit IgG (1:5000 dilution) conjugated to horseradish peroxidase (GE Healthcare Bio-Sciences, Little Chalfont, Buckinghamshire, UK), and the ECL Plus reagent (GE Healthcare Bio-Sciences). Protein determinations were performed using reagents from Bio-Rad Laboratories (Hercules, CA).

19-HETE in Mouse Sera. Quantification of 19-HETE was performed using a liquid chromatography/tandem mass spectrometry method adapted from a published method (Newman et al., 2002). On-line liquid chromatography of extracted samples was performed with Agilent Technologies 1100 Series capillary high-performance liquid chromatography. Separations were achieved using a Phenomenex (Torrance, CA) Luna C18(2) column (5 μm, 150 × 2 mm), which was held at 40°C. The flow rate was 350 μl/min. Mobile phase A was 0.1% acetic acid in water. Mobile phase B was 0.1% acetic acid in 85:15 acetonitrile/methanol. Gradient elution was used, and the mobile phase percentage B was varied as follows: 15% B at 0 min, ramp to 2 min to 30% B, ramp from 2 to 5 min to 55% B, and ramp from 5 to 25.5 min to 75.5% B. Samples were spiked with 30 ng of 10,11-dihydroxynonadecanoic acid (10,11-DiHN) in 10 μl of ethanol as an internal standard before extraction. 10,11-DiHN was supplied by John Newman (University of California, Davis, CA). 19-HETE for external calibration was supplied by John R. Falck (University of Texas Southwestern, Dallas, TX). Dried sample extracts were reconstituted in 100 μl of 50% ethanol. Triplicate injections of 20 μl were analyzed.

Electrospray ionization/tandem mass spectrometry was used for detection. Analyses were performed on an Applied Biosystems/MD Sciex (Foster City, CA) API 3000 equipped with a TurboIonSpray source. Turbo desolvation gas was heated to 350°C at a flow rate of 7 l/min. All the analytes were monitored as negative ions with the instrument in multiple-reaction monitoring mode. Analyses were monitored at the following parent ion–product ion mass/charge ratio pairs and retention times (tR): 19-HETE, 319.2–275.0 (tR = 16.8 ± 0.1 min); and 10,11-DiHN, 329.2–311.2 (tR = 18.7 ± 0.1 min).

Results

Induction of CYP2C55 mRNA in Livers and Kidneys. Car(+/+) and Car(−/−) mice were given intravenous injections of saline or PB (100 mg/kg) and sacrificed 24 h later. The mRNA levels in these mouse livers were determined by real-time reverse transcription (RT)-PCR. The PB treatment increased hepatic CYP2C55 mRNA 140-fold in Car(+/+) but not in Car(−/−) mice (Fig. 1A). Likewise, the hepatic CYP2C55 mRNA was examined in Pxr(+/-) and Pxr(−/-) mice intraperitoneally treated with DMSO or PCN (20 mg/kg). The hepatic CYP2C55 mRNA was induced more than 140-fold in Car(+/-) and Car(−/-) mice treated with DMSO or PCN (20 mg/kg). The hepatic CYP2C55 mRNA was induced more than

![Fig. 1. CAR- and PXR-dependent induction of hepatic CYP2C55 mRNA in mice. A and B, mice were given intravenous injections of saline, PB (100 mg/kg, i.v.), intraperitoneal DMSO, or PCN (20 mg/kg, i.p.) and sacrificed 12 and 24 h after injection, respectively. Relative mRNA levels in these mouse livers were determined by real-time RT-PCR and were expressed by taking those in the Car(+/-) or Pxr(+/-) mice treated with vehicle as equal to one. Values express mean ± S.D. (n = 3). **, p < 0.01 for vehicle-injected group versus drug-injected group in the Car(+/-) and Car(−/-) mice.](https://example.com/fig1.png)

![Fig. 2. CAR- and PXR-dependent induction of renal CYP2C55 mRNA in mice. A, mice were given intravenous injections of saline or PB (100 mg/kg) and sacrificed 12 h after the injection. B, mice were given intraperitoneal injections of DMSO or PCN (20 mg/kg) and sacrificed 24 h after the injection. Relative mRNA levels in these mouse kidneys were determined by real-time RT-PCR and were expressed by taking those in the Car(+/-) or Pxr(+/-) mice treated with vehicle as equal to one. Values express mean ± S.D. (n = 3). **, p < 0.01 for vehicle-injected group versus drug-injected group in the Car(+/-) and Pxr(+/-) mice.](https://example.com/fig2.png)
15-fold in Pxr(+/+) mice treated with PCN but not in Pxr(−/−) mice (Fig. 1B). Next we examined the effect of chronic PB treatment on the hepatic CYP2C55 mRNA. Car(+/+) and Car(−/−) mice were given a single intraperitoneal dose of DEN (90 mg/kg) and treated chronically with PB (500 ppm) for 6 weeks. The hepatic CYP2C55 mRNA was induced approximately 30-fold in Car(+/+) mice treated with PB for 6 and 32 weeks but not in Car(−/−) mice (Fig. 1, C and D).

Car(+/+) and Car(−/−) mice received intravenous injections of saline or PB (100 mg/kg) and were sacrificed 12 h after the injection. Renal CYP2C55 mRNA levels were induced 50-fold in Car(+/+) mice treated with PB but not in Car(−/−) mice (Fig. 2A). Likewise, renal CYP2C55 mRNA levels were examined in Pxr(+/+) and Pxr(−/−) mice treated with DMSO or PCN (20 mg/kg) at 24 h after the injection. Renal CYP2C55 mRNA was induced more than 4-fold only in the Pxr(+/+) mice (Fig. 2B).

Microsomal CYP2C55 in Mouse Livers. Car(+/+) and Car(−/−) mice were given a single intraperitoneal dose of DEN (90 mg/kg) and treated chronically with PB (500 ppm) for 6 weeks. Western blot analysis was performed with liver microsomes prepared from these mice. Hepatic CYP2C55 protein was clearly increased in Car(+/+), but not in Car(−/−) mice (Fig. 3). In addition, we confirmed that the hepatic CYP2B10 protein was also only induced in the Car(+/+) mice by PB.

Serum 19-HETE Level in Mice. Car(+/+) mice (three mice for each group) were given a single intraperitoneal dose of DEN (90 mg/kg) and treated chronically with PB (500 ppm) for 6 weeks. Serum 19-HETE levels in these mice were measured by liquid chromatography/tandem mass spectrometry analysis. The serum 19-HETE level was significantly induced 2-fold by PB treatment: 2.5 ± 0.5 and 5.5 ± 1.5 for DEN + PB treatment versus DEN treatment, respectively, p < 0.05.

A CAR/PXR Response Element within the Cyp2c55 Promoter. SeqLab GcG (Accelrys, San Diego, CA) was used to search a 10-kb DNA sequence of the Cyp2c55 5′-flanking region for a CAR/PXR response element, an imperfect direct repeat of AGGTCA spaced by three to five nucleotides (DR-n). Two putative response elements were found upstream of the Cyp2c55 5′-flanking region, including a DR4 motif (−1679/−1664) and a DR5 motif (−669/−653) (Fig. 4). Luciferase reporters containing various lengths of the Cyp2c55 5′-flanking region were constructed and cotransfected with CAR or PXR into Huh7 cells for transient transfection assays. Whereas the Cyp2c55−1.6 kb luciferase reporter was not activated by the CAR ligand 1.4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), the Cyp2c55−2.5 kb luciferase reporter was activated 8-fold (Fig. 5A). This Cyp2c55−2.5 kb luciferase reporter was also activated 17- and 25-fold by PCN when PXR was cotransfected (Fig. 5B). To determine the role of the DR4 site (−1679/−1664) in this activation, this site was deleted from the Cyp2c55−2.5 kb luciferase reporter. Neither TCPOBOP nor PCN activated the DR4-deleted promoter (Fig. 5, C and D). Furthermore, gel shift assays confirmed specific binding of CAR and PXR to the DR4 sequence (data not shown).

Discussion

CYP2C subfamily enzymes are known to metabolize xenochromics and endogenous compounds such as AA. Mouse CYP2C55 was characterized as an enzyme that preferentially catalyzes the biosynthesis of 19-HETE (Wang et al., 2004), an AA metabolite known to...

![Figure 3](image3.png)

**Fig. 3.** CAR-dependent induction of hepatic CYP2C55 protein in mice. (three per group) were given a single intraperitoneal dose of DEN (90 mg/kg) and chronically treated with PB (500 ppm) for 6 weeks. Western blot analysis was performed with liver microsomes prepared from these mice. Hepatic CYP2C55 protein was clearly increased in Car(+/+) mice by PB treatment but not in Car(−/−) mice (Fig. 3). In addition, we confirmed that the hepatic CYP2B10 protein was also only induced in the Car(+/+) mice by PB.

![Figure 4](image4.png)

**Fig. 4.** Alignment of CYP2C55 5′-flanking region. A schematic representation of the Cyp2c55 5′-flanking region provides the putative CAR and PXR binding sites.

![Figure 5](image5.png)

**Fig. 5.** Transcriptional activation analysis of the Cyp2c55 promoter by mCAR and mPXR in Huh7 cells. A and B, reporter plasmid, pGL3/Cyp2c55−2.5 kb and pGL3/Cyp2c55−1.6 kb, were cotransfected with or without pCR3/mCAR and pcDNA3.1/mPXR as indicated. At 24 h after transfection, cells were treated with DMSO, TCPOBOP (250 nM), and PCN (10 μM) and incubated for an additional 24 h. Relative luciferase activities were expressed by taking the activity of the DMSO-treated cells transfected with the −2.5-kb reporter plasmid alone as equal to one. C and D, reporter plasmid, pGL3/Cyp2c55−2.5 kb, and the internal deletion mutants of the putative binding sites, DR4 and DR5, were transfected with or without pCR3/mCAR and pcDNA3.1/mPXR as indicated. At 24 h after transfection, cells were treated with DMSO, TCPOBOP (250 nM), and PCN (10 μM) and incubated for an additional 24 h. Relative luciferase activities were expressed by taking the activity of the DMSO-treated cells transfected with the −2.5-kb reporter plasmid alone as one.
have potent physiological effects such as effects on renal vascular tone and ion transport (Escalante et al., 1988; Ma et al., 1993; Carroll et al., 1996). Herein, we found that CAR and PXR regulate drug induction of CYP2C55 mRNA in mouse liver and kidney by using CYP2C55 knock-out mice. Furthermore, we also found that chronic PB treatment increased serum 19-HETE levels. Because other P450 isozymes (e.g., CYP2E1, CYP4A, CYP2C, and CYP2F2) (Laethem et al., 1993; Luo et al., 1998; Nguyen et al., 1999; Qu et al., 2001; Poloyac et al., 2004; Wang et al., 2004; Cowpland et al., 2006) are known to synthesize 19-HETE, the actual contribution of CYP2C55 to the PB-induced 19-HETE remains to be determined in future investigations. 19-HETE has been suggested to be vasodilated renal artery (Ma et al., 1993) and stimulate the renal cortical Na+/K+ ATPase (Escalante et al., 1988) and proximal tubule transporters (Quigley et al., 2000). Although there have been no reports that chronic usage of CAR and PXR activators such as PB and rifampicin resulted in alteration of renal function, this might be an area of future research. Moreover, it is of interest that among the human CYP2C enzymes, CYP2C19 is unique in that it produces primarily 19-HETE from AA (Bylund et al., 1998). Moreover, CYP2C19 is inducible by CAR and PXR (Chen et al., 2003). It is noteworthy that in the studies in Cyp3a-null mice that CYP2C55 was markedly induced and that the authors also suggested that food-derived xenobiotics might up-regulate CYP2C55 (van Waterschoot et al., 2008). Additional studies by the same group (van Waterschoot et al., 2009) showed that the CAR ligand TCBOBOP induced CYP2C55. Dexamethasone-induced CYP2C55 in wild-type mice but not PBX knockout mice. There were differences in CYP2C55 expression in mice being fed with semisynthetic versus commercial chow, for which the mechanism was not investigated further.

Drug-induced transcriptional regulation of the human CYP2C subfamily genes, such as CYP2C9, by CAR and PXR has been characterized (Ferguson et al., 2002; Gerbal-Chaloin et al., 2002; Chen et al., 2004; Ferguson et al., 2005). Within the mouse CYP2C subfamily, the Cyp2c29 and Cyp2c37 genes were reported to be up-regulated by CAR but not by PXR (Jackson et al., 2004, 2006). Herein, we identified a functional CAR promoter and PXR responsive element (−1679/−1464) within the Cyp2c55 promoter. Cyp2c55 is the first murine Cyp2c gene that has been shown to be regulated by CAR. Both CAR and PXR bind to the same responsive element DR4 (−1679/−1664) and activate the Cyp2c55 gene in liver and kidney. Induction of Cyp2c55 may be responsible for the increase of 19-HETE levels in serum of DEN-PB-treated mice.

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References


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Address correspondence to: Masahiko Negishi, Pharmacogenetics Section, Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC 27709. E-mail: negishi@niehs.nih.gov